## Specific Example of How In Vitro Assays Have Been Used at RJRT as Part of a Toxicological Evaluation Program

### Eclipse

R J Reynolds has developed a cigarette that primarily heats tobacco. Under the FTC puffing regimen, the mainstream smoke from this cigarette is reduced significantly in a number of chemical compounds when compared with other cigarettes of similar "tar" delivery (Borgerding *et al.*, 1998). The mainstream particulate matter (CSC) has been evaluated in the neutral red cytotoxicity, Ames, SCE and chromosome aberration assays, and the whole smoke (vapor phase plus particulate) has been evaluated in the SCE and neutral red cytotoxicity assay (Bombick, B. *et al.*, 1998; Bombick D. *et al.*, 1998). The genotoxic potential of cigarette smoke condensate from cigarettes that primarily heat tobacco is significantly reduced compared to that of cigarettes that burn tobacco, evidenced by significant reduction in the induction of Ames bacterial mutagenicity, chromosomal aberrations, and sister chromatid exchanges. The data from these studies are presented in Figures 3 through 10.

### Summary

- RJRT has actively pursued the development of cigarettes with the potential to reduce risks by:
  1. Continuing to pursue general and specific smoke constituent reductions in tobacco-burning cigarettes.
  2. Continuing to develop and refine alternative cigarette designs.
  3. Finding ways to alter "tar"/nicotine ratio.
  4. Ensuring no product modifications add to the biological activity.
- RJRT has developed and applied assays to evaluate the progress of cigarette modifications.
- A regulatory framework should be designed that allows free-market forces to develop products that have the potential to reduce risks.
- A tiered testing strategy for toxicological evaluation has been used at RJRT to determine the level and degree of testing required which include chemical and biological assays.
- Short-term in vitro assays are a subset of the biological assays which have been developed or applied by RJRT with success for use on cigarette smoke and cigarette smoke condensate.

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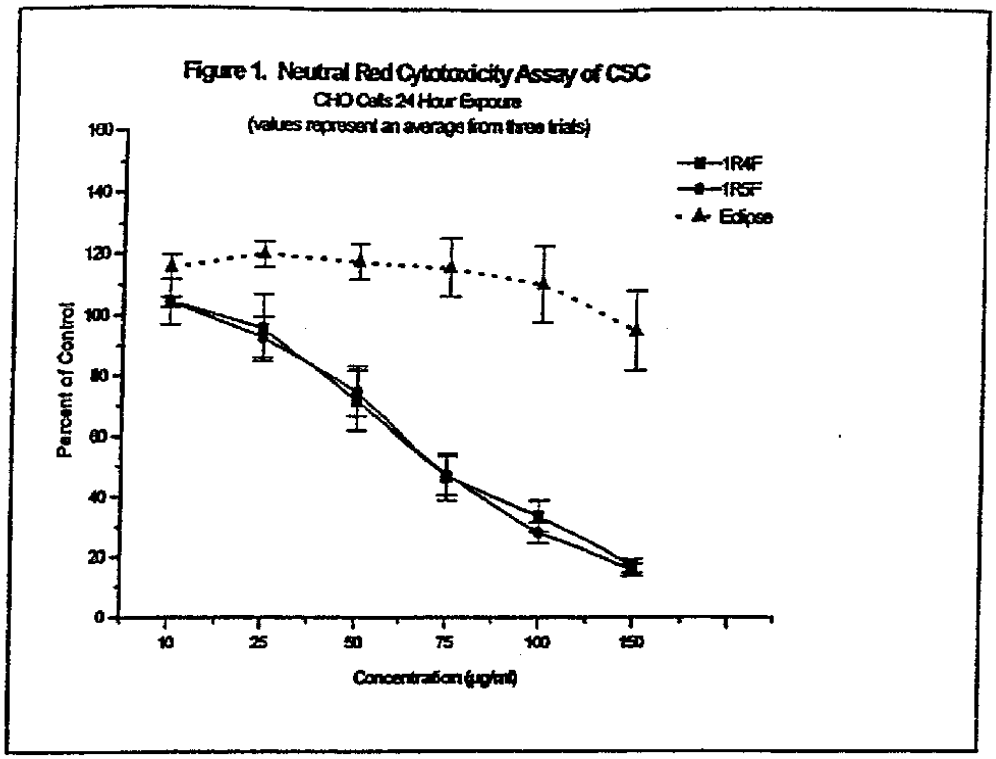


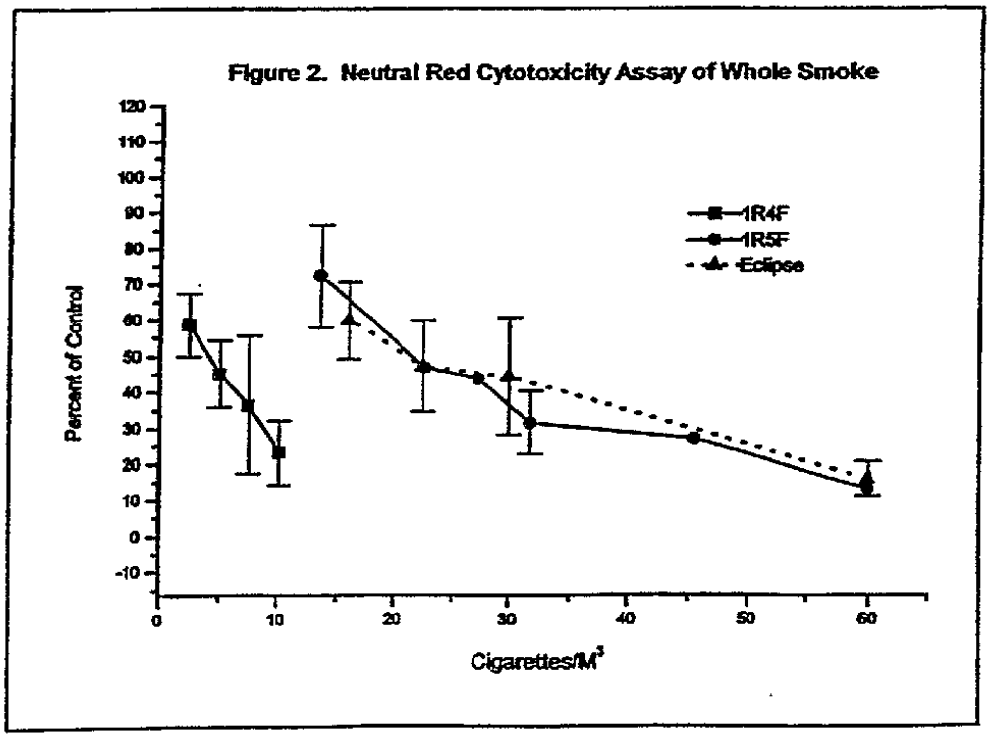

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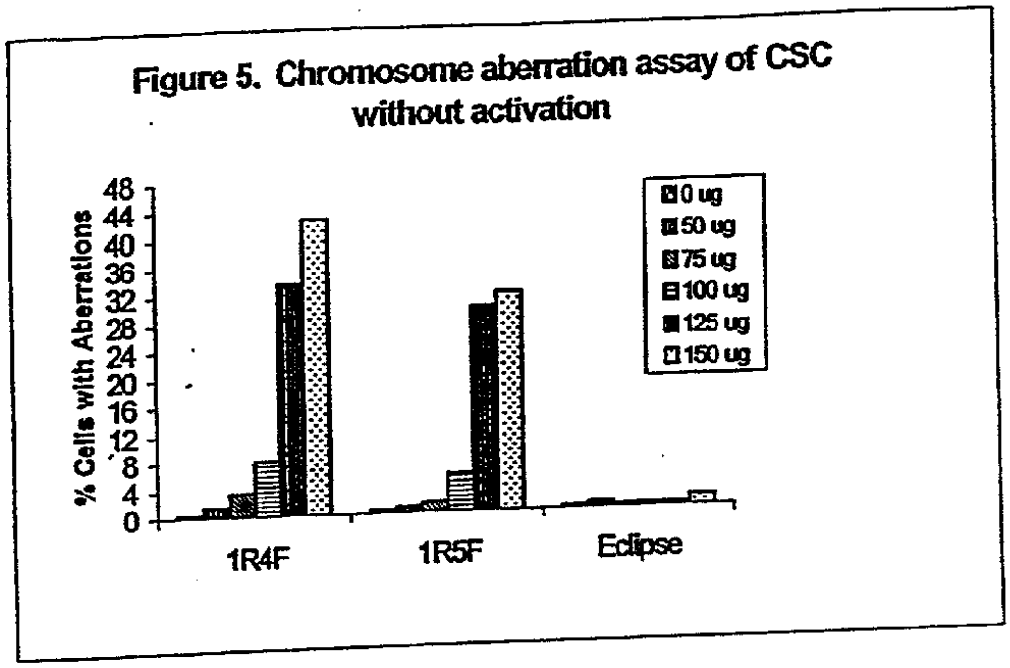


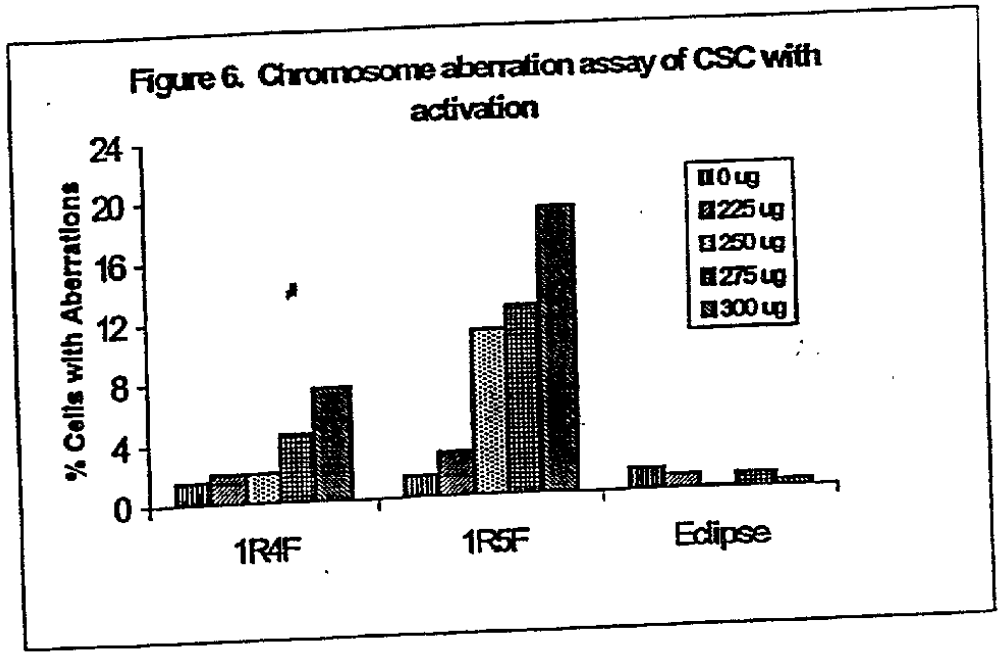

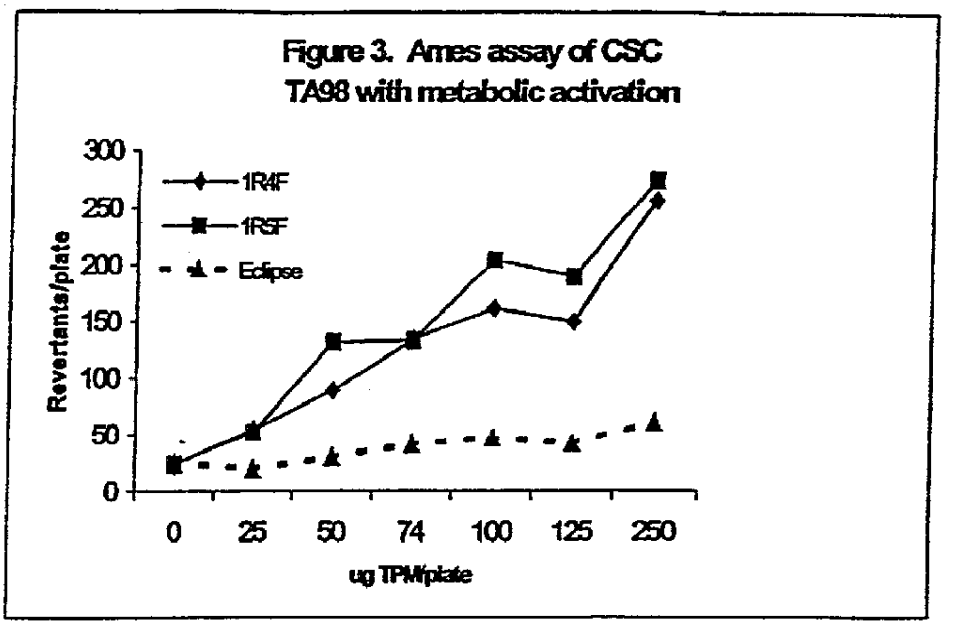


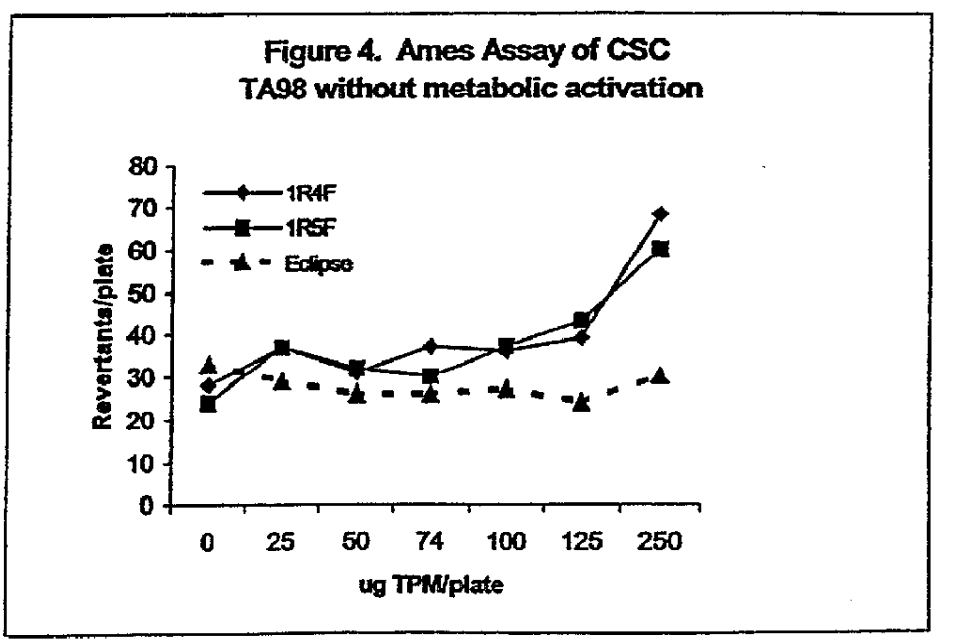

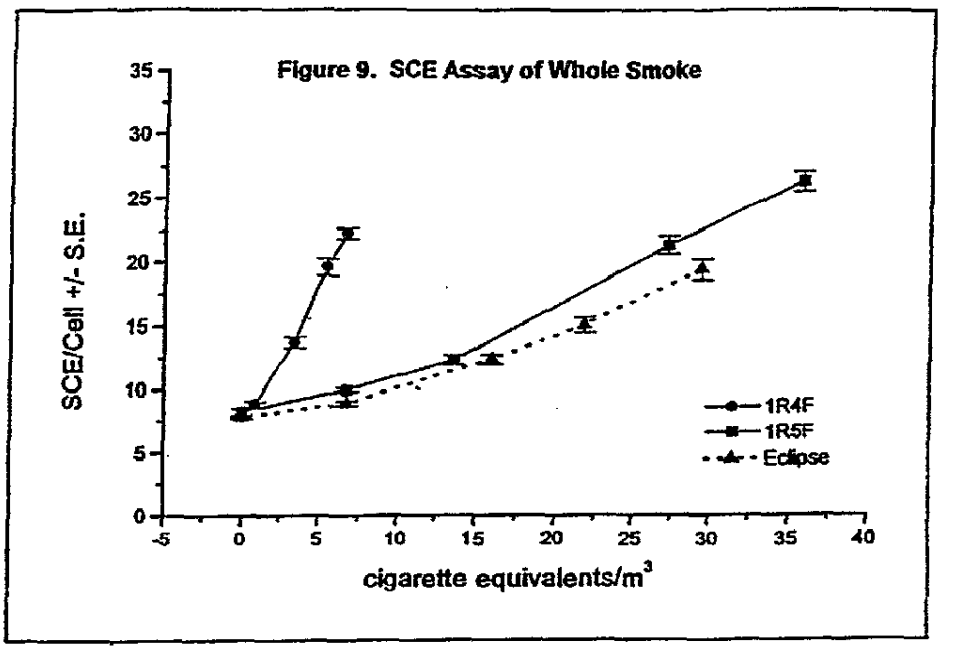


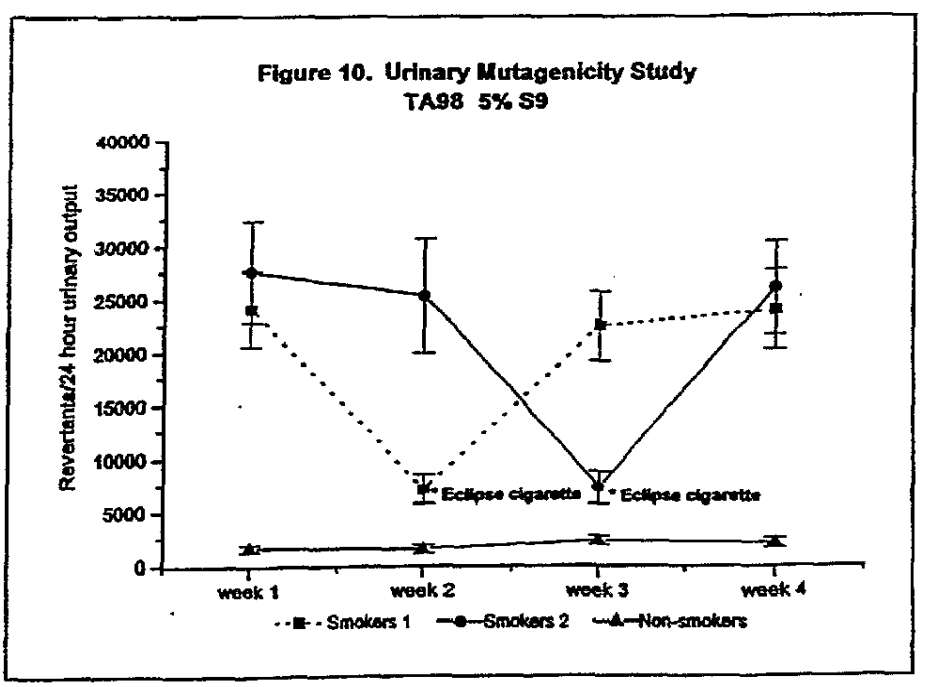

Figure 7. SCE assay of CSC without activation

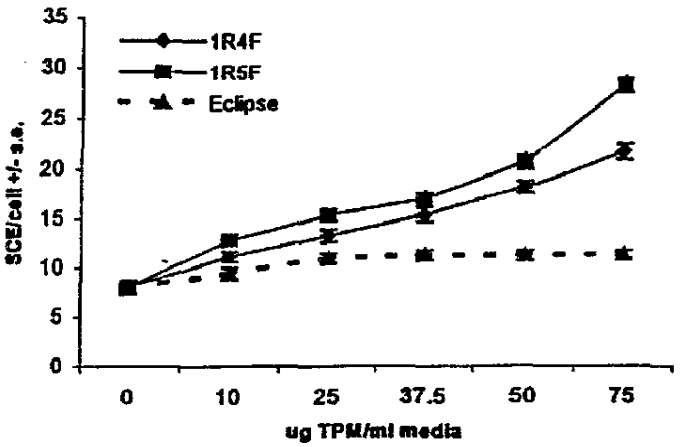


Figure 8. SCE Assay of CSC with activation

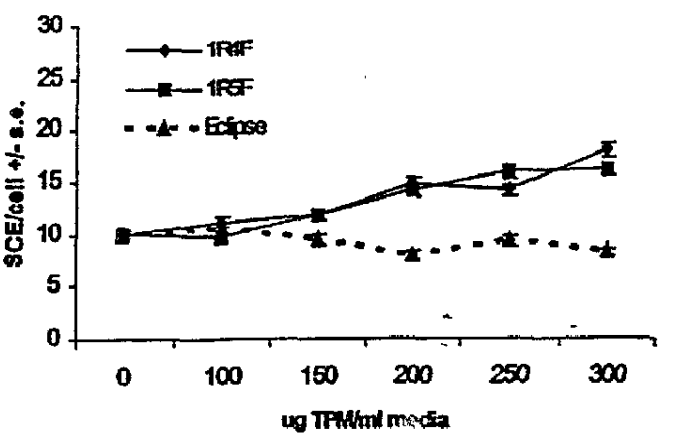

**Figure 5.** **Chromosome aberration assay of three cigarette smoke condensates without metabolic activation.** The Chromosome aberration assay was conducted in Chinese Hamster Ovary (CHO) cells according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. CHO cells were exposed to CSC for two hours and harvested 20 hours after initiation of treatment. Concentration is expressed as ug Total Particulate Matter (TPM) per ml of media.

**Figure 6.** **Chromosome aberration assay of three cigarette smoke condensates in the presence of metabolic activation.** The Chromosome Aberration assay was conducted in Chinese Hamster Ovary (CHO) cells in the presence of S9 metabolic activation according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. CHO cells were exposed to CSC for two hours in the presence of a S9 metabolic activation system, and harvested 20 hours after the initiation of treatment. Concentration is expressed as ug Total Particulate Matter (TPM) per ml media.

**Figure 7.** **SCE Assay of three cigarette smoke condensates without metabolic activation.** The Sister Chromatid Exchange (SCE) assay was conducted in Chinese Hamster Ovary (CHO) cells according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. CHO cells were exposed to CSC for approximately twenty-four hours and harvested approximately 26-29 hours after initiation of treatment. Concentration is expressed as ug Total Particulate Matter (TPM) per ml media.

## Legend

**Figure 1.** **Assessment of cytotoxic potential of three cigarette smoke condensates using the Neutral Red assay.** The cytotoxic potential of the smoke condensate of Eclipse (a cigarette which primarily heats tobacco) was compared with smoke condensates from low 'tar' 1R4F and 1R5F ultra-low 'tar' Reference cigarettes. Smoke condensates were prepared under a standard FTC puffing regimen of 35-ml puffs of two-second duration every minute. Concentration is expressed as ug total particulate matter per mL of cell culture medium. The cytotoxic response is expressed as a percentage of control (cells exposed to solvent vehicle). CHO (Chinese Hamster Ovary) cells were used in the cytotoxicity assessment with an exposure time of 24 hours. For complete details see Bombick, B.R. *et al.*, 1998.

**Figure 2.** **Assessment of cytotoxic potential of the whole smoke from three cigarettes using the Neutral Red assay.** The cytotoxic potential of whole smoke from Eclipse (a cigarette which primarily heats tobacco) was compared with the whole smoke from low 'tar' 1R4F and ultra-low 'tar' 1R5F reference cigarettes. Smoke was generated from a 30-port AMESA smoke generator under a FTC puffing regimen of 35-ml puffs of 2-second duration every minute. Concentration is expressed as the number of cigarettes per cubic meter. The cytotoxic response is expressed as a percentage of control (cells exposed to humidified room air). Chinese Hamster Ovary (CHO) cells were exposed to smoke for one hour and cytotoxicity assessed after 24 hours. For complete details see Bombick, D.W. *et al.*, 1998.

**Figure 3.** **Ames assay of three cigarette smoke condensates with TA98 without metabolic activation.** The Ames assay was conducted in the preincubation assay according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. Concentration is expressed as ug Total Particulate Matter (TPM) per plate.

**Figure 4.** **Ames assay of three cigarette smoke condensates with TA98 in the presence of metabolic activation.** The Ames assay was conducted in the preincubation assay in the presence of S9 metabolic activation according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low "tar" cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. Concentration is expressed as ug Total Particulate Matter (TPM) per plate.

**Figure 8.** **SCE assay of three cigarette smoke condensates in the presence of metabolic activation.** The Sister Chromatid Exchange assay was conducted in Chinese Hamster Ovary (CHO) cells in the presence of S9 metabolic activation according to procedures described in Bombick, B.R. *et al.*, 1998. Cigarette Smoke Condensates (CSC) were prepared under a standard FTC puffing regimen of 35 ml puffs of two-second duration every minute. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. CHO cells were exposed to CSC for two hours in the presence of a S9 metabolic activation system, and harvested approximately 29-31.5 hours after the initiation of treatment. Concentration is expressed as ug Total Particulate Matter (TPM) per ml media.

**Figure 9.** **SCE Assay of Whole Smoke.** The Sister Chromatid Exchange (SCE) assay was conducted in Chinese Hamster Ovary (CHO) cells according to procedures described in Bombick, D.W. *et al.*, 1998. Smoke was generated from a 30-port AMESA smoke generator under a FTC puffing regimen of 35 ml puffs of 2-second duration every minute. Concentration is expressed as the number of cigarettes per cubic meter. The three CSCs tested were Eclipse, a cigarette that primarily heats tobacco; 1R4F, a Kentucky Reference low 'tar' cigarette, and 1R5F, a Kentucky Reference ultra-low 'tar' cigarette. CHO cells were exposed to smoke for one hour and harvested 27.5 to 32 hours after the initiation of treatment.

**Figure 10.** **Human Urinary Mutagenicity Study using TA98 5% S9.** The Ames assay was conducted by the microsuspension modification according to procedures described in Smith, *et al.*, 1996. Twenty-two smokers and 12 nonsmokers were enrolled in a 4-week single crossover study. Each smoker consumed Eclipse cigarettes *ad libitum* for a week and their usual brand of cigarettes the other 3 weeks. A strictly controlled diet was administered to minimize ingestion of mutagenic protein pyrolysis products. 24-hour urine samples were collected weekly, concentrated using XAD-2 resin, and tested in the microsuspension Ames assay with TA98 in the presence of S9 metabolic activation. Mutagenicity is expressed as the number of revertants per 24 hour-urinary output.